PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Confirmation No. 3724
J. Kevin DONAHUE	)	Group Art Unit: 1636
Serial No. 09/977,865	)	Examiner: K. Katcheves
Filed: October 15, 2001	)	
For: **Methods and Compositions for Nucleic Acid Delivery**	)	Atty. Docket No. 001107.00449

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, J. Kevin Donahue, declare:

1. I am a named co-inventor of the application named above. I am an Associate Professor of Medicine at The Johns Hopkins University School of Medicine in Baltimore, Maryland in the Division of Cardiology.

2. Following the teachings of the subject application, we have successfully delivered nucleic acids to cells in a tissue by perfusion through vasculature of a tissue of interest in whole animals. A cGMP-specific phosphodieterase-5 (PDE-5) inhibitor was administered as a part of the procedure.

3. We used intracoronary perfusion to deliver nucleic acids to ten pigs that had been given oral sildenafil, a PDE-5 inhibitor. Gene transfer and expression was detected in 45% of the cells of the atrioventricular (AV) node. Other organs were found to express the transferred gene in less than 1% of the cells. When a gene encoding the

$G\alpha_{i2}$ subunit of inhibitory G protein was transferred to the heart by this method, a 20% reduction in ventricular rate during atrial fibrillation was observed. Donahue *et al.*, "Focal modification of electrical conduction in the heart by viral gene transfer," *Nature Medicine*, 6:1395-1398 (2000); Exhibit 1.

4. We administered PDE-5 inhibitor sildenafil *per os* to domestic swine. Bauer *et al.*, "Inhibitory G Protein Overexpression Provides Physiologically Relevant Heart Rate Control in Persistent Atrial Fibrillation," *Circulation*, 110:3115-3120 (2004); Exhibit 2. Viral DNA encoding inhibitory G protein alpha-subunit (either wild-type or constitutive mutant) was infused via a catheter into the atrioventricular (AV) nodal artery. The heart rate of the animals that received the constitutively active mutant progressively decreased over the first few days, followed by a stable 15-25% reduction in heart rate for the remainder of the study period. See Exhibit 2, Fig. 2. We also observed that the administration of the constitutively active mutant reduced apoptosis in the left and right ventricles. See Exhibit 2, Fig. 5.

5. These studies demonstrate that the delivery of genes via *in vivo* perfusion of target tissues successfully transfers genes to the cells and successfully provides polypeptides to tissues of interest.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12/16/04

J. Kevin Donahue, M.D.

EXHIBIT I

Focal modification of electrical conduction in the heart by viral gene transfer

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Modern treatment of cardiac arrhythmias is limited to pharmacotherapy, radiofrequency ablation, or implantable devices. Antiarrhythmic medications suppress arrhythmias, but their systemic effects are often poorly tolerated and their proarrhythmic tendencies increase mortality[1–3]. Radiofrequency ablation can cure only a limited number of arrhythmias. Implantable devices can be curative for bradyarrhythmias and lifesaving for tachyarrhythmias, but require a lifetime commitment to repeated procedures, are a significant expense, and may lead to severe complications. One possibility is the use of gene therapy as an antiarrhythmic strategy. As an initial attempt to explore this option, we focused on genetic modification of the atrioventricular node. First, we developed an intracoronary perfusion model for gene delivery, building on our previous work in isolated cardiac myocytes and hearts perfused *ex vivo*[4,5]. Using this method, we infected porcine hearts with Adβgal (recombinant adenovirus expressing *Escherichia coli* β-galactosidase) or with AdG_i (adenovirus encoding the $G\alpha_{i2}$ subunit). We hypothesized that excess $G\alpha_{i2}$ would mimic the effects of β-adreneric antagonists, in effect creating a localized β-blockade. $G\alpha_{i2}$ overexpression suppressed baseline atrioventricular conduction and slowed the heart rate during atrial fibrillation without producing complete heart block. In contrast, expression of the reporter gene β-galactosidase had no electrophysiological effects. Our results demonstrate the feasibility of using myocardial gene transfer strategies to treat common arrhythmias.

In previous *ex vivo* and *in vitro* studies, we found that gene transfer efficiency correlated with coronary flow rate, virus exposure time, virus concentration and the level of microvascular permeability[4,5]. We also found that elimination of radiographic contrast media and red blood cells from the perfusate and delivery at body temperature were necessary for optimal results. The *in vivo* delivery system used here builds on those findings.

We subjected ten animals to a protocol that included medication with oral sildenafil before baseline electrophysiology (EP) study, catheterization of the right coronary artery and infusion of vascular endothelial growth factor (VEGF), nitroglycerin and virus-containing solutions (7.5×10^9 p.f.u. in 1 ml) into the atrioventricular (AV) nodal branch of the right coronary artery. VEGF was used to increase microvascular permeability[6], and sildenafil potentiated the VEGF effect. The infusion volume and coronary flow rate were limited to avoid efflux from the artery and infection of other regions of the heart. Five animals received Adβgal, and the other five received AdG_i. The animals underwent follow-up EP study seven days after virus infusion. After the second EP study, the hearts were explanted and evaluated for β–galactosidase (β-gal) and $G\alpha_{i2}$ expression. Other adenoviral gene transfer studies have shown that expression is detectable after 3 days, peaks after 5–7 days and then regresses over 20–30 days[7–9]. Based on these data, we tested for gene expression and phenotypic changes seven days after gene delivery.

X-gal staining revealed β-gal activity in the AV nodal region and adjacent ventricular septum of all Adβgal-infected animals (Fig. 1*a*). There was no evidence of β-gal activity in any of the AdG_i-infected animals or in other heart sections from the Adβgal group. Microscopic sections through the AV node showed gene transfer to 45 ± 6% of the AV nodal cells in the Adβgal group and confirmed the absence of X-gal staining from the AdG_i-infected animals. We also observed a mild inflammatory infiltrate, composed mainly of mononuclear cells.

Western-blot analysis was performed on tissue homogenates from the AV nodal region of four animals from each group (Fig. 1*b*). Densitometry analysis confirmed $G\alpha_{i2}$ overexpression in the AdG_i group, amounting to a 500% increase in $G\alpha_{i2}$ relative to the Adβgal animals ($P = 0.01$). The level of $G\alpha_{i2}$ in the Adβgal group was not different from that found in two uninfected control animals (data not shown).

We performed X-gal staining of gross and microscopic sections from lungs, liver, kidney, skeletal muscle and ovaries of all animals to evaluate the extent of gene transfer outside the heart (Fig. 1*c*). In the Adβgal-infected animals, β-gal activity was evident in gross specimens from liver, kidneys and ovaries. No staining was evident in lungs or skeletal muscle (data not shown). Microscopic sections revealed β-gal activity, but in less than 1% of the cells in these organs. We observed no X-gal staining in any tissues of the AdG_i-infected animals or uninfected control animals. The lack of X-gal staining in AdG_i-infected and uninfected controls indicates that the results were specific for transgene expression and not from endogenous β-gal activity or false-positive staining. These results are consistent with a previous study documenting gene expression in peripheral organs after intracardiac injection of adenovirus[10].

We obtained EP measurements at baseline and seven days after infection (Table 1). Electrocardiogram (ECG) parameters were taken from the surface ECG, and the AH and HV intervals were recorded from an intracardiac catheter in the His-bundle position. (These intervals indicate the speed of conduction through

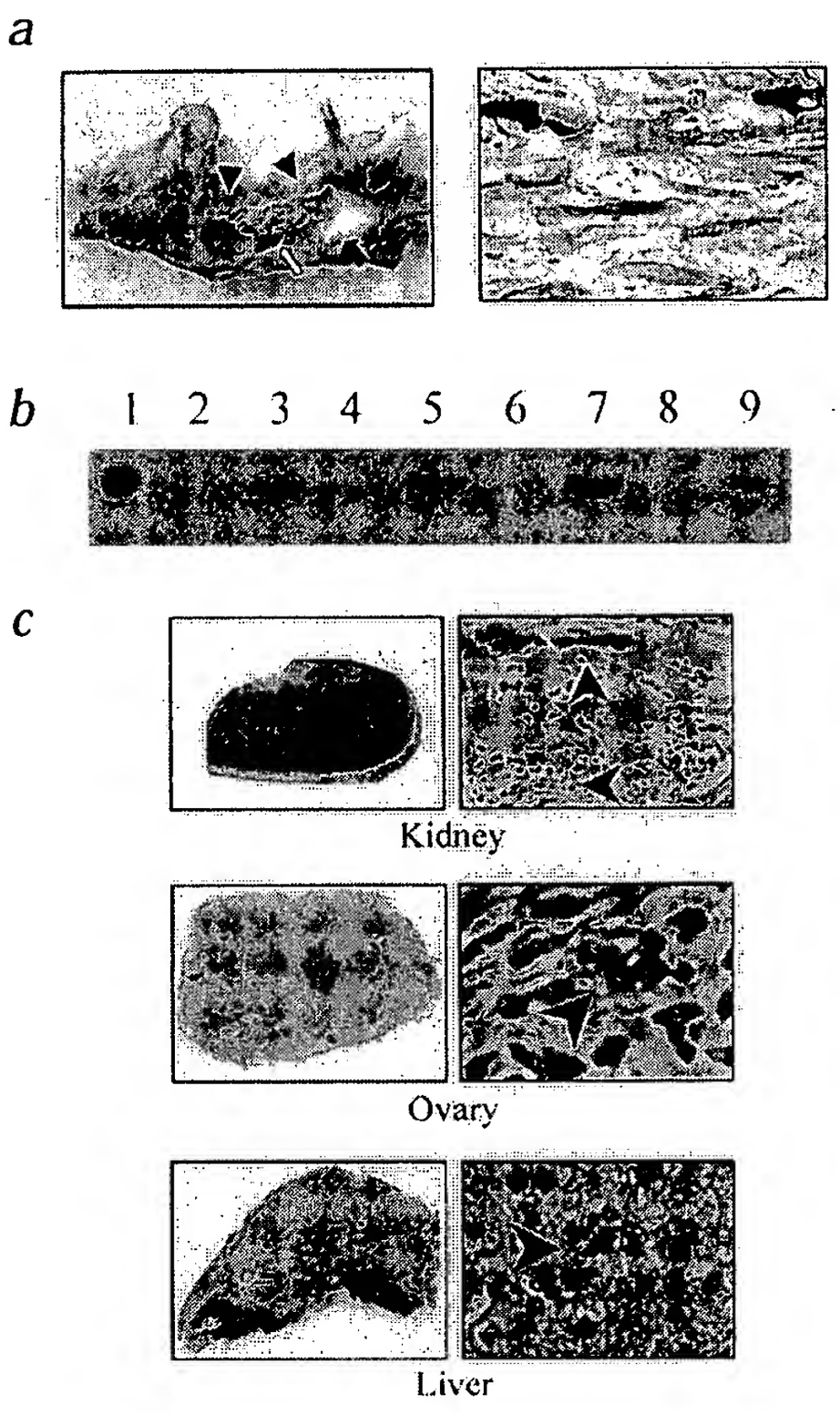

Fig. 1 Measurement of gene transfer efficacy. ***a***, X-gal staining of a transverse section through the AV groove. Arrowheads indicate the tricuspid valve ring, and the filled arrow marks the central fibrous body. The open arrow points to the AV node. A microscopic section through the AV node shows gene transfer to 45 ± 6% of myocytes. Cells expressing β-gal are stained blue. ***b***, Western blot of AV nodal tissue demonstrates $G\alpha_{i2}$ overexpression in the AdG_i infected animals. Lane 1, 10 μg $G\alpha_{i2}$ control; lanes 2, 4, 6, 8, Adβgal-infected animals; lanes 3, 5, 7, 9, AdG_i-infected animals. Analysis of the bands shows a 500 ± 100% increase in $G\alpha_{i2}$ content in the AdG_i animals relative to the Adβgal-infected controls. ***c***, Gross and microscopic pathology after exposure of liver, kidney and ovary to X-gal solution. Microscopic sections show rare blue cells (arrowheads) in these organs.

various regions of the heart. The AH interval measures conduction through the AV node, and the HV interval evaluates conduction distal to the AV node, through the His-Purkinje system to the ventricles.) We measured the AV node effective refractory period (AVNERP) by pacing the atria at a stable rate for eight beats and then delivering premature atrial stimuli at progressively shorter intervals, noting the interval where the premature beat failed to conduct through the AV node. There were no significant differences in the EP parameters between groups at baseline. In the Adβgal group, comparison of baseline measurements with those taken seven days after infection also failed to show any significant differences. In contrast, the follow-up study of the AdG_i group revealed significant prolongation in the PR interval on the surface ECG (paired analysis: day 0, 97 ± 2 ms; day 7, 109 ± 4 ms; $P = 0.01$), the AH interval on the intracardiac electrogram (day 0, 60 ± 2 ms; day 7, 76 ± 3 ms; $P = 0.01$) and the AVNERP (day 0, 226 ± 6 ms; day 7, 246 ± 3 ms; $P = 0.03$), indicating both slowed conduction and an increased refractory time in the AV node after $G\alpha_{i2}$ overexpression.

After measurement of basic EP intervals, we measured the heart rate during acute episodes of atrial fibrillation (Fig. 2*a*). Overexpression of $G\alpha_{i2}$ in the AV node caused a 20% reduction in the ventricular rate during atrial fibrillation (day 0, 199 ± 5 b.p.m.; day 7, 158 ± 2 b.p.m., $P = 0.005$). This effect persisted in the setting of adrenergic stimulation. Administration of epinephrine (1 mg, intravenous injection) increased the atrial fibrillation heart rate in all animals, but the group overexpressing $G\alpha_{i2}$ had a 16% reduction in ventricular rate (day 0; 364 ± 3 b.p.m.; day 7, 308 ± 2 b.p.m.; $P = 0.005$). In contrast, β-gal expression did not affect the heart rate during atrial fibrillation, either before (day 0, 194 ± 8 b.p.m.; day 7, 191 ± 7 b.p.m.; P = NS) or after epinephrine administration (day 0, 362 ± 6 b.p.m.; day 7, 353 ± 5; P = NS).

To further evaluate the effect of $G\alpha_{i2}$ overexpression on AV conduction, we analyzed the heart rate at various time points after induction of atrial fibrillation in the AdG_i-epinephrine group (Fig. 2*b*). These data indicate that the ventricular rate remains stable and that the beneficial suppression of heart rate from $G\alpha_{i2}$ gene transfer is sustained through at least three minutes of observation. The episodes of atrial fibrillation often lasted longer than three minutes (see methods), but the period of observation was limited to ensure that the effects of epinephrine would be constant.

We chose $G\alpha_{i2}$ to suppress conduction because of the success of β-blocking drugs at achieving that goal. In the AV node, β-adrenergic receptors are coupled to stimulatory G proteins (G_s). Stimulation of β-receptors activates G_s, releasing the $G\alpha_s$-subunit to stimulate adenylate cyclase[11]. This process leads to a cascade of intracellular events causing an increase in conduction velocity and a shortening of the refractory period. β-blockers prevent the increase in AV nodal conduction by inhibiting receptor activation.

The intracellular processes responsive to G_s are counterbalanced by the activity of inhibitory G proteins (G_i). In the AV node, G_i couple with muscarinic M2 and adenosine A1 receptors[11]. G_i activation releases the $G\alpha_i$-subunit to bind and inhibit adenylate cyclase activity. The $G\beta\gamma$-subunit is also released to increase potassium conductance by direct action on acetylcholine-activated potassium channels. The cumulative effect of G_i

Table 1 EP parameters before and 7 days after gene transfer

		Adβgal		AdG_i	
Day		0	7	0	7
Heart rate during sinus rhythm		114 ± 5	111 ± 1	113 ± 2	106 ± 4
ECG:	PR interval	101 ± 1	99 ± 1	97 ± 2	109 ± 5*
	QRS interval	58 ± 2	54 ± 1	57 ± 1	56 ± 1
	QT interval	296 ± 6	310 ± 2	288 ± 7	316 ± 6
AH interval		61 ± 1	61 ± 1	60 ± 2	76 ± 3*
HV interval		25 ± 1	25 ± 1	23 ± 1	24 ± 1
AVNERP		226 ± 6	224 ± 4	226 ± 6	246 ± 3*

mean ± s.e.m., $n = 5$ in each group. * $P \leq 0.03$

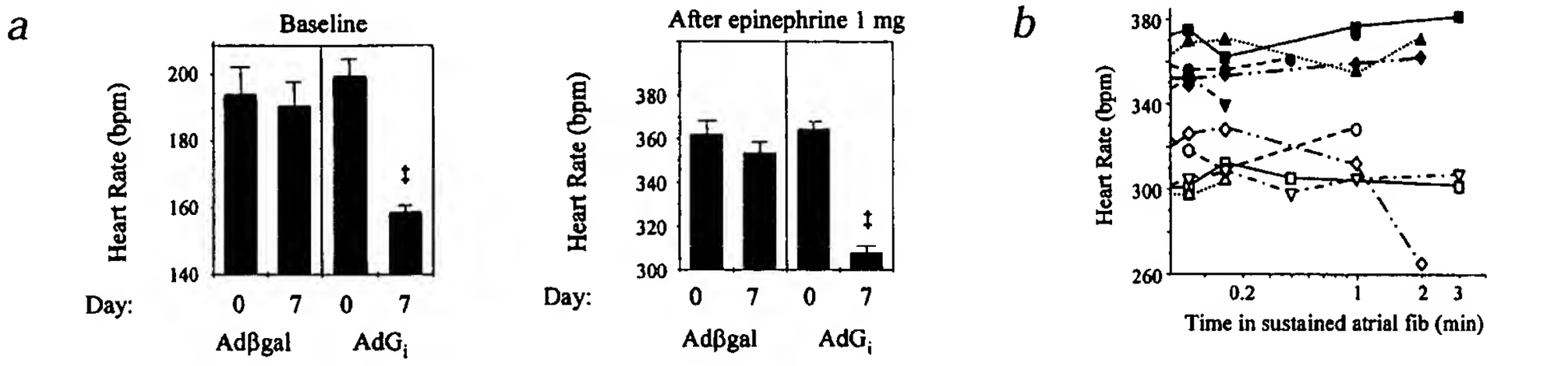

Fig. 2 Reduction in heart rate during atrial fibrillation after $G\alpha_{i2}$ gene transfer. ***a***, $G\alpha_{i2}$ overexpression reduces ventricular rate by 20% during atrial fibrillation in the drug-free state. No difference in heart rate is observed after Adβgal exposure. After infusion of epinephrine (1 mg IV), the relative effect of $G\alpha_{i2}$ overexpression persists. (‡ $P = 0.005$). ***b***, In the AdG_i group, the heart rate was measured at several time points after epinephrine bolus and induction of atrial fibrillation. Each animal is represented by a unique line and symbol. ■,●,▲,◆,▼ are from day 0, before gene transfer; □,○,△,◇,▽ are from day 7, after gene transfer.

activation is a decrease in conduction through the AV node. In agreement with these known effects of the G_i cascade, our data show that overexpression of $G\alpha_{i2}$ suppresses AV nodal conduction in the drug-free state and during adrenergic stimulation.

Under ordinary circumstances, $G\alpha_{i2}$-mediated inhibition of adenylate cyclase requires receptor activation[12]. But here, G_i activity seems to be uncoupled from the receptor, because the inhibition occurs without exogenous M2 or A1 receptor stimulation. In the setting of 500% overexpression of $G\alpha_{i2}$, normal cellular mechanisms might be altered. Further study will be required to elucidate the changes in signal transduction that underlie the observed effects.

A principal focus of our study was to overcome the problem of vector delivery to the myocardium using minimally invasive techniques. By manipulation of the tissue and vascular dynamics, the genes encoding β-gal and $G\alpha_{i2}$ were transferred to 45% of AV nodal myocytes by intracoronary catheterization. A limited inflammatory response was noted after adenoviral infection, but there was no detectable effect on AV nodal function from the inflammation or from reporter gene transfer. Other studies have shown that the use of first-generation adenoviruses (those with deletions at E1) leads to intense inflammation and loss of transgene expression 20 to 30 days after infection[13]. When used at high concentrations (much greater than those used here), adenovirus vectors are also associated with endothelial damage, arterial thrombosis, thrombocytopenia, anemia, hepatitis and death[14-17]. Wild-type adenoviruses have also been implicated in the development of myocarditis and idiopathic cardiomyopathy[18]. Because we used a relatively low concentration of virus and looked at phenotypic changes early after gene transfer, these limitations did not affect our findings.

This study is the first report of intracoronary site-specific gene transfer and the first use of gene therapy to treat cardiac arrhythmias. We have demonstrated that overexpression of an inhibitory component of the β-adrenergic signaling pathway suppresses AV nodal conduction, and documented the absence of EP changes after adenovirus-mediated transfer of a reporter gene. Our research shows that *in vivo* gene transfer can modify the cardiac electrical substrate implicated in common arrhythmias.

Methods

Adenoviruses. Adβgal was a gift from F. Graham; the vector contained the *Escherichia coli lacZ* gene driven by the human cytomegalovirus immediate early promoter. AdG_i was constructed as described[19]. The vector included the full-length rat $G\alpha_{i2}$ gene driven by the cytomegalovirus promoter. Virus stock expansion and quality control were performed as described[4].

Gene transfer procedure. Immediately before catheterization, female domestic swine (30–40 kg) received sustained-release 180 mg diltiazem, 325 mg aspirin and 25 mg sildenafil orally, and a mixture of 100 kg ketamine and 4 mg acepromazine intramuscularly. For uniformity, the same pretreatment regimen, except administration of sildenafil, was used for all procedures to control for any effect these agents might have on the baseline EP measurements. After sedation, anesthesia was induced with 5–10 ml of intravenous sodium pentothal 2.5% solution and maintained with inhaled isoflurane 2% in oxygen. The right carotid artery, right internal jugular vein and right femoral vein were accessed by sterile surgical technique, and introducer sheaths were inserted into each vessel. After baseline EP study, the right coronary artery was catheterized via the right carotid artery, using a 7 F angioplasty guiding catheter. The AV nodal branch was selected with a 0.014-inch guide wire, over which a 2.7 F infusion catheter was inserted into the AV nodal artery. The following solutions were infused through the catheter: 10 ml normal saline (NS) containing 5 μg $VEGF_{165}$ and 200 μg nitroglycerin over 3 minutes, 1 ml NS containing 7.5×10^9 p.f.u. adenovirus and 20 μg nitroglycerin over 30 s, and 2 ml NS over 30 s. After recovery from anesthesia, the animals received usual care and no additional medication. After one week, repeat EP evaluation was performed, the animals were killed and the organs were removed for histological evaluation. The animals used here were maintained in accordance with the guiding principles of the American Physiological Society regarding experimental animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

Electrophysiological evaluation. Immediately before gene transfer and one week afterward, the animals underwent EP evaluation. A 5 F steerable quadripolar EP catheter was placed through the right internal jugular vein into the high right atrium; a 5 F non-steerable quadripolar EP catheter was placed through the same internal jugular vein into the right ventricle, and a 6 F non-steerable quadripolar EP catheter was placed through the right femoral vein into the His- bundle position. Baseline intracardiac electrograms were obtained, and electrocardiographic intervals were recorded. Following standard techniques, the AVNERP was measured by programmed stimulation of the right atrium with a drive train cycle length of 400 ms.

After baseline measurements were obtained, atrial fibrillation was induced by burst atrial pacing from a cycle length of 180 ms decrementing to 100 ms over 30 s. Three attempts were made using this induction protocol. If no sustained atrial fibrillation was induced, the atria were paced at an output of 10 mA and a cycle length of 20 ms for 15 s. The latter protocol reliably induced atrial fibrillation. The first episode of atrial fibrillation lasting longer than 12 s was used for analysis. The median duration for atrial fibrillation episodes was 20 s (range 14–120 s). The heart rate was determined by measuring R–R intervals during the first 10 seconds of atrial fibrillation (average number of R–R intervals measured was 32 per recording). After conversion back to sinus rhythm, 1 mg of epinephrine was administered

through the femoral venous sheath. Atrial fibrillation was re-induced in the presence of epinephrine (median episode duration 131 s, range 20–600 s), and the heart rate was again measured (average number of R–R intervals measured was 60 per recording). In the drug-free state, all episodes of atrial fibrillation terminated spontaneously. After epinephrine infusion, 4 episodes persisted for 10 min and were terminated by electrical cardioversion.

Histological evaluation. After the animals were killed, the heart and sections of lung, liver, kidney, skeletal muscle and ovary were removed and rinsed thoroughly in PBS. The atrial and ventricular septa were dissected from the heart and frozen to –80 °C. The remaining portions of the heart and other organs were sectioned, and alternating sections were used for gross or microscopic analysis. The sections for gross examination were fixed in 2% formaldehyde/0.2% glutaraldehyde for 15 min at room temperature, and stained for 6 h at 37 °C in PBS containing 1.0 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 15 mmol/l potassium ferricyanide, 15 mmol/l potassium ferrocyanide and 1 mmol/l $MgCl_2$. After staining, the slices were fixed with 2% formaldehyde/0.2% glutaraldehyde in PBS at 4 °C overnight. The sections for microscopic analysis were embedded in paraffin, cut to 7 μm thickness, stained with X-gal solution as above and counterstained with hematoxylin and eosin stains using traditional methods. β-gal expression in the AV node was quantified by counting 100 cells in randomly chosen high-power fields of microscopic sections through the region.

Western-blot analysis of $G\alpha_{i2}$ expression. Analysis of $G\alpha_{i2}$ protein expression was performed on cytosolic extracts of frozen AV nodal tissue (Novex System). Samples were normalized for protein content, and SDS–PAGE of the normalized samples was performed on 4–12% gradient gels. Proteins were then transferred to nitrocellulose membranes (30 V, 1 h). Detection of protein was performed by sequential exposure to western blocking reagent (Boehringer), a mouse monoclonal antibody against $G\alpha_{i2}$ (Neomarkers, 1 Ïg/ml, 2 h), and goat secondary antibody against mouse conjugated with horseradish peroxidase (NEN, 1:10000, 30 min). Bands were detected with the enhanced chemiluminescence assay (Amersham) and quantified using the Quantity One software package (BioRad).

Statistical analysis. The data are presented as mean ± s.e.m. Statistical differences were determined using Student's *t*-test and repeated measures ANOVA, where appropriate. A P value of < 0.05 was considered statistically significant.

Acknowledgements

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EXHIBIT II

Inhibitory G Protein Overexpression Provides Physiologically Relevant Heart Rate Control in Persistent Atrial Fibrillation

Alexander Bauer, MD; Amy D. McDonald, BS; Khurram Nasir, MD; Leah Peller, BS; Jeffrey J. Rade, MD; Julie M. Miller, MD; Alan W. Heldman, MD; J. Kevin Donahue, MD

Background—The need for new treatment strategies for cardiac arrhythmias has motivated our continuing development of gene therapeutic options. Previously, we reported a decreased heart rate in an acute model of atrial fibrillation after atrioventricular nodal gene transfer. Here, we expand those observations to persistent atrial fibrillation and severe heart failure.

Methods and Results—After 3 weeks of atrial fibrillation, domestic swine received atrioventricular nodal gene transfer with adenoviruses encoding β-galactosidase (β-gal), wild-type $G\alpha_{i2}$ (wtGi), or constitutively active mutant (cGi). Heart rates in awake, alert animals were not altered by β-gal or wtGi. cGi caused a sustained 15% to 25% decrease in heart rate. The wtGi effect became evident with sedation. A tachycardia-induced cardiomyopathy was present before gene transfer. In the β-gal group, cardiomyopathy worsened over time. In the wtGi group, the condition improved slightly, and in the cGi group, ejection fraction was near normal at the end of the study. TUNEL staining results corroborated this finding.

Conclusions—cGi overexpression in the porcine atrioventricular node causes physiologically relevant heart rate control in persistent atrial fibrillation. These data advance the development of gene therapy as a potential treatment for common cardiac arrhythmias. **(*Circulation*. 2004;110:3115-3120.)**

Key Words: arrhythmia ■ gene therapy ■ electrophysiology ■ atrioventricular node ■ fibrillation

Several large clinical trials have associated antiarrhythmic drug therapy with increased mortality,[1,2] demonstrating a need for other options to treat cardiac arrhythmias. Ablation and implantable devices are useful for several applications, but neither modality fully solves the problem. Radiofrequency ablation can cure focal arrhythmias (eg, atrioventricular [AV] node reentry tachycardia or accessory pathway–mediated tachycardia), but ablation only palliates more diffuse arrhythmias such as atrial fibrillation (AF) or infarct-related ventricular tachycardia.[3,4] Implantable devices unquestionably save lives, but devices are associated with significant expense, potential complications from implant and replacement procedures, and in the case of defibrillators, pain related to the shock therapy.

AFFIRM and RACE are the most recent clinical trials to refute the antiarrhythmic drug paradigm. These trials compared the strategies of rhythm and rate control for treatment of AF.[5,6] Like most antiarrhythmic trials, the bias going into these studies was that rhythm control (ie, maintenance of sinus rhythm with drugs or electrical cardioversion) would be proven superior to continued AF with control of the ventricular rate. The ultimate results demonstrated no benefit to the antiarrhythmic drug strategy and a suggestion of harm from the antiarrhythmic drugs, this time a nonsignificant trend toward increased deaths in the rhythm control arm of AFFIRM.[5]

We recently reported a gene therapy strategy for rate control in AF.[7] That proof-of-concept report documented an 18% heart rate reduction during acute AF in instrumented, anesthetized pigs after gene transfer of an inhibitory G protein α-subunit ($G\alpha_{i2}$). Questions arising from those results included the applicability to persistent AF in awake, alert animals and the significance of the observed 18% heart rate reduction. The current work answers those concerns. Using a previously reported model of persistent AF and heart failure,[8] we evaluated the effects of AV nodal gene transfer with wild-type $G\alpha_{i2}$ (wtGi) and a constitutively active mutant $G\alpha_{i2}$ Q205L (cGi). Because gene expression is known to be limited with first-generation adenoviral (Ad) vectors, the physiologic observations in this report are limited to an 18-day window when Ad-mediated gene expression is known to be stable.[9]

Methods

Adenoviruses

Ad–β-galactosidase (Adβ-gal) and AdwtGi were provided by Frank Graham (McMaster University, Hamilton, ON, Canada) and Thomas

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Eschenhagen (University Hospital Eppendorf, Hamburg, Germany), respectively.[7] AdcGi was constructed using the Cre-Lox system as previously reported.[10] Quality control of virus stocks included virus concentration determination by DNA absorbance, infective particle titer by plaque assay, transgene expression confirmation by Western blot analysis after transduction of HeLa cells, and absence of replication-competent virus by polymerase chain reaction analysis.

Chronic AF Model

Persistent AF was induced using atrial burst pacing as previously described.[8,11] Domestic swine (18 to 22 kg) were sedated with ketamine 100 mg/kg and anesthetized with pentothal (2 to 5 mL of a 5% solution) and isoflurane (1% to 2%). A pacing lead (Medtronic) was fixed in the right atrial appendage and connected to a pacemaker in the right neck (Medtronic). The atria were paced at 42 Hz for 2-second intervals whenever the atrial rate fell below 180 beats/min. For purposes of this study, persistent AF (as opposed to paroxysmal AF) was defined as continuous AF without any evidence of sinus rhythm on the daily ECG tracings.

Throughout the study, clinical observations and ECG recordings were performed on a daily basis with a 6-lead ECG system. Animals were awake and alert at consistent levels from one reading to the next. Clinical observations included spontaneous activity level, appetite, evidence of dyspnea or edema, and overall appearance. The animals for this study were maintained in accordance with the guiding principles of the American Physiological Society regarding experimental animals. The experimental protocol was approved by the Johns Hopkins Institutional Animal Care and Use Committee.

Gene Transfer Procedure

On postpacemaker day 21 (gene transfer day 0), the animals underwent coronary catheterization for gene delivery. Domestic swine received 325 mg aspirin and 25 mg sildenafil PO and 1000 mg ketamine IM. Anesthesia was induced with 1000 mg IV ketamine and maintained with inhaled isoflurane, 0.5% to 2% in oxygen. The right coronary artery was catheterized, and the AV nodal branch was identified as the vessel tracking in the direction of the AV node originating near the posterior descending coronary artery. All animals in this study had right-dominant coronary systems with a single obvious vessel in the position of the AV nodal branch, so no further maneuvers were undertaken to identify the vessel. A 2.7F infusion catheter was inserted over a guidewire into the AV nodal artery. The following solutions were infused: 10 mL of Krebs' solution containing 5 μg vascular endothelial growth factor$_{165}$ and 500 μg nitroglycerin over 3 minutes; 1 mL of Krebs' solution containing 1×10^{10} plaque-forming units of Ad and 20 μg nitroglycerin over 30 seconds; and 2 mL of normal saline over 20 seconds. A standard randomization scheme was used to determine which virus was used in each animal. The investigator responsible for clinical observations and ECG measurements was blinded to the virus randomization scheme.

Echocardiographic Examinations

Echocardiographic examinations were performed at pacemaker implantation, gene transfer, and on post–gene transfer day 14. All measurements were performed after activation of the pacing protocol for consistent heart rate and irregularity. Left ventricular ejection fractions were calculated from parasternal short- and long-axis views, and chamber sizes were determined from M-mode images. All measurements were made using American Society of Echocardiography criteria.[12]

Histologic Evaluation

After euthanization by intravenous KCl overdose in fully anesthetized animals, hearts were removed, and sections for microscopic analysis were fixed in 10% formalin, embedded in paraffin, cut to 7-μm thickness, and stained with hematoxylin and eosin or Masson's trichrome by traditional methods. Terminal dUTP nick end-labeling (TUNEL) staining was performed according to standard protocols.[13] Negative controls were incubated with label solution only, and sections incubated with DNAse I (Sigma) served as positive controls.

Sections were examined at $\times$64 magnification in a random order and blinded fashion by 2 observers. The reported histologic score is the average of these observations. Variables included nuclear enlargement, cellular hypertrophy, cellular myolysis, interstitial fibrosis, and interstitial inflammation. Each sample was graded on a scale of 1 to 5 as previously reported.[8]

Statistical Analysis

The data are presented as mean$\pm$SEM. Heart rate data were controlled for repeated measurements and to determine correlations between subjects by use of a generalized estimating equation (GEE) with an exchangeable working relation to analyze the longitudinal relations of heart rate to potential use of different modes of therapy. For each pig i, the heart rate measured daily y_i (y_1, y_2, y_3, y_4, . . . y_{18}) was modeled by the XTGEE procedure of the STATA computer package. This method fits a regression model between heart rate measurements and effect of therapy for each pig, taking into account the inherent variability of slope estimates from individual pigs.[14]

For the more straightforward analyses, statistical differences were determined using Student's t test and repeated-measures ANOVA, where appropriate. A probability value <0.05 was considered statistically significant.

Results

Baseline Observations Before Gene Transfer

Details of the ventricular response rate and resulting tachycardia-induced cardiomyopathy have been previously reported.[8] Like the animals in that report, those in the current study had nonsustained episodes of AF immediately after activation of the burst-pacing algorithm and continuous AF by day 5$\pm$1. After developing continuous AF, the animals had no further recurrences of sinus rhythm for the duration of the study. Heart rate was measured once per day in awake, eating animals. During burst pacing and nonsustained AF, the ventricular rate was 281$\pm$12 beats/min on the first day after implantation of the pacemaker. Over the first 7 days, the heart rate in AF was 271$\pm$4 beats/min, and over the 21-day period before gene transfer, the average rate was 274$\pm$4 beats/min (P=NS). There were no significant differences in ventricular rate comparing intervals of burst atrial pacing, paroxysmal AF, and persistent AF.

Immediately after initiation of the burst-pacing protocol, 2 animals developed syncope resulting from the sudden increase in heart rate. These animals recovered without intervention and had no further episodes of syncope. Otherwise, there were initially no apparent behavioral changes resulting from the rapid heart rate. After 14 days, the animals displayed nonspecific signs of lethargy, increased sleep, mild dyspnea with exertion of walking from the pen to the ECG recording/feeding area, decreased play habits, and decreased appetite. Weight gain over the 3-week period averaged 1.3$\pm$0.1 kg/wk. In contrast, a set of normal control pigs did not display these behavioral changes, and weight gain averaged 1.8$\pm$0.3 kg/wk ($P<0.01$). Echocardiograms showed significant changes in cardiac dimensions and ventricular contractility comparing day 0 with day 21 (Table). There were no statistically significant differences between groups at the time of gene transfer (day 21).

Absence of Rate Control From AV Node–Blocking Drugs in the Porcine AF Model

To evaluate the efficacy of conventional drugs in this model, we tested the effects of digoxin, diltiazem, and esmolol

Echocardiographic Measurements

Group	Parameter	Day of PM	Day of GT	14 Days After GT
β-gal	LA	1.7±0.1	2.6±0.2*	3.4±0.1*
	LVEDD	1.8±0.1	3.6±0.2†	4.1±0.1*
	RVEDD	1.5±0.1	2.2±0.2†	2.7±0.2*
	LVST	0.8±0.1	0.7±0.1	0.7±0.2
	LVPT	0.8±0.1	0.6±0.1	0.6±0.1
	LVEF	72±4	29±2†	22±3*
wtGi	LA	1.4±0.1	3.0±0.2†	3.5±0.2*
	LVEDD	1.8±0.2	4.0±0.3†	4.1±0.2
	RVEDD	1.9±0.3	2.7±0.1†	2.8±0.2
	LVST	0.6±0.1	0.7±0.1	0.7±0.1
	LVPT	0.6±0.1	0.6±0.1	0.6±0.1
	LVEF	71±4	24±3†	31±3*
cGi	LA	1.6±0.1	2.4±0.2†	2.5±0.2
	LVEDD	1.9±0.2	4.1±0.2†	3.6±0.3
	RVEDD	1.9±0.1	2.3±0.1†	2.4±0.2
	LVST	0.8±0.1	0.7±0.1	0.9±0.1
	LVPT	0.8±0.1	0.5±0.1	0.7±0.1*
	LVEF	69±3	34±5†	46±4†

PM indicates pacemaker implantation; GT, gene transfer; LA, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; RVEDD, right ventricular end-diastolic diameter; LVST, left ventricular septal thickness; LVPT, left ventricular posterior wall thickness; and LVEF, left ventricular ejection fraction.
*$P<0.05$.
†$P<0.01$.

administration on ventricular rate. Groups of 5 animals each received sustained-release diltiazem (6 mg/kg) or digoxin (6.3 μg/kg) daily for 5 days. Both doses were chosen because they would be relatively large human doses. Neither agent had any significant effect (Figure 1). On postpacemaker day 21, 4 sedated animals received intravenous esmolol (100 μg/kg), a short-acting β-blocker, given at the weight-based recommended human dose. The reduction in heart rate was negligible after esmolol administration (Figure 1).

In each case, the drug dose was limited by toxicity. Both esmolol and diltiazem worsened heart failure. In the diltiazem-treated animals, dyspnea and edema increased progressively during the 5-day course of therapy. Administration of esmolol caused intractable pulmonary edema and contractile failure leading to death despite aggressive intervention in all 4 animals. None of these animals received AV nodal gene transfer, so they were not included in any further analysis. Digoxin-treated animals had no observable positive or negative effects during drug therapy. Digoxin serum level was found to be in a range considered toxic for humans (3.2±0.3 ng/dL), so the dose was not increased further.

Differential Effects of cGi and wtGi on Heart Rate Reduction in AF

On day 21 after pacemaker implantation, 15 animals underwent the AV nodal gene transfer procedure. Groups of 5 animals each received AdwtGi encoding the wild-type rat

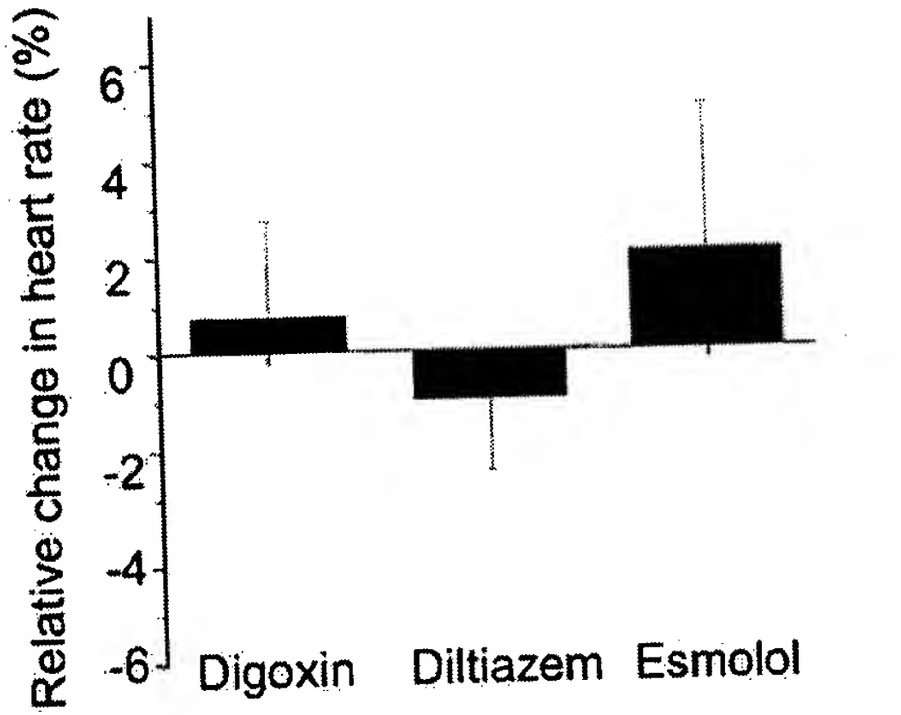

Figure 1. Relative changes in heart rate after administration of sustained-release diltiazem (6 mg/kg) or digoxin (6.3 μg/kg) daily for 5 days or after short-term administration of esmolol (single dose of 100 μg/kg IV bolus). None of these conventional AV node–blocking agents caused a significant decrease in heart rate.

$G\alpha_{i2}$, AdcGi containing the constitutively active mutant mouse $G\alpha_{i2}$ Q205L,[15] or Adβ-gal with *Escherichia coli* β-gal. We have previously shown that Adβ-gal gene transfer does not affect AV nodal function,[7] so the Adβ-gal animals were included as gene transfer controls for comparison with the 2 G-protein groups. Because gene expression is known to be limited with first-generation Ad vectors, the physiologic observations in this report are limited to an 18-day window when Ad-mediated gene expression is known to be stable.[9]

Heart rate was measured on a daily basis (Figure 2). From the GEE model, we noted a significant effect over time. Pigs with higher baseline heart rates sustained their higher heart rates after therapy relative to other animals within each group ($P<0.001$), and no effect of obtaining repeated measurements daily ($P=0.38$) was observed. The β-gal control group did not change significantly from baseline. The cGi group had a progressive decrease in heart rate over the first few days, followed by a stable 15% to 25% reduction in heart rate for

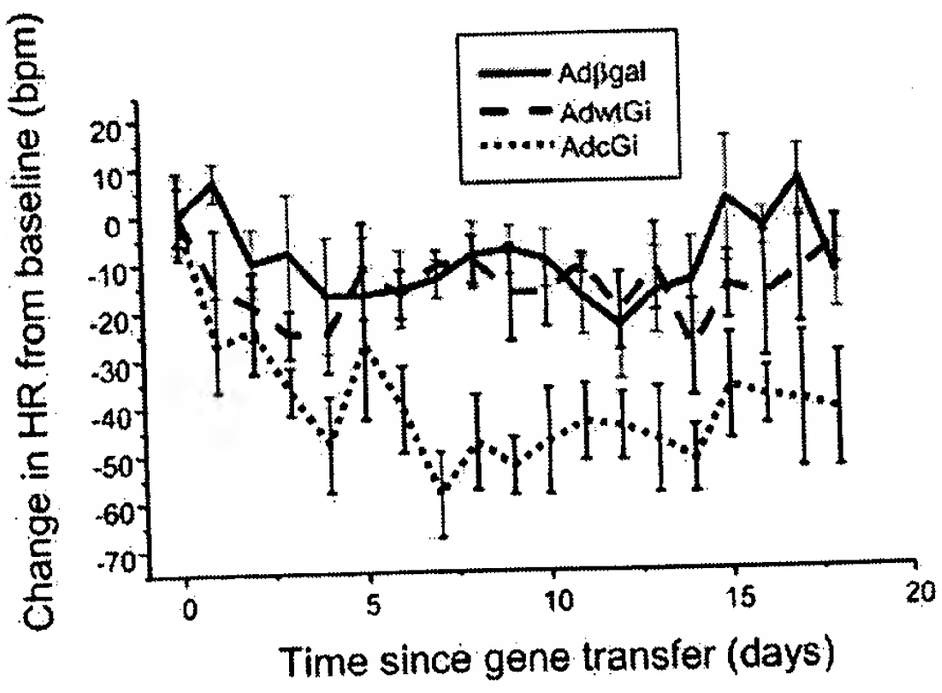

Figure 2. Change in heart rate over time after gene transfer. Abbreviations are as defined in text.

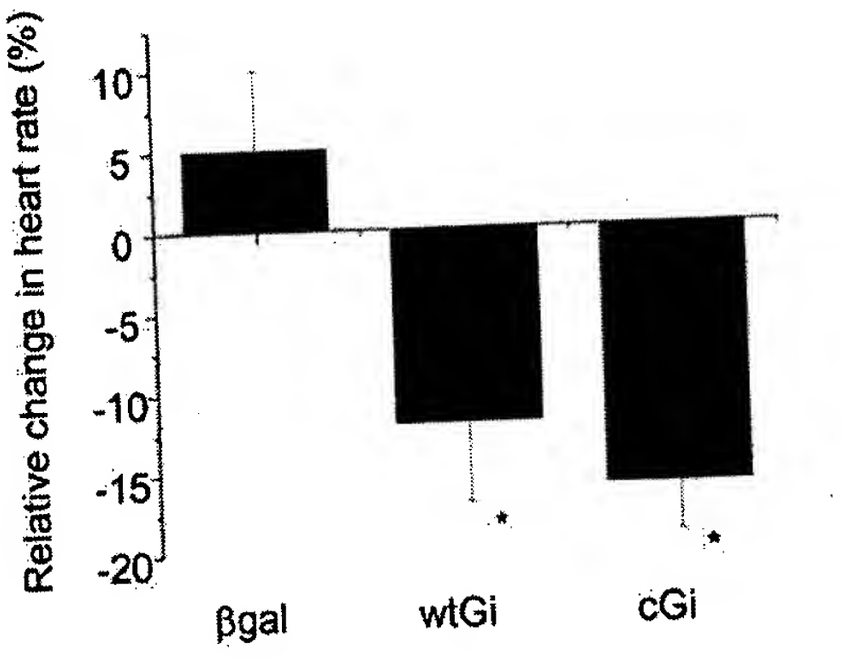

Figure 3. Relative changes in heart rate after sedation. Comparison is made of heart rate on day of gene transfer and on post–gene transfer day 7. *$P<0.01$, day 7 vs day 0. Abbreviations are as defined in text.

the remainder of the study ($P<0.001$ compared with either wtGi or β-gal). Surprisingly, the wtGi animals had no significant change in heart rate when compared with the Adβ-gal controls ($P=0.1$). All animals remained in AF for the duration of the study, and no heart block or ventricular arrhythmias were noted in any animals during this study. All animals survived to the conclusion of the study, and there were no strokes, myocardial infarctions, or sudden deaths observed.

Etiology of Reduced wtGi Effects in Chronic AF Compared With Acute AF

Our observed results in chronic AF contrasted the previous experience in acute AF.[7] To investigate this anomaly, we re-created the conditions used in the previous report by evaluating heart rate during sedation on the seventh day after gene transfer. The results demonstrate that wtGi effects are elicited by sedation (Figure 3). Ad-cGi–infected animals had a 16±3% reduction in heart rate when comparing post–gene transfer day 0 with day 7 ($P<0.01$); Ad-wtGi animals had a 12±5% reduction ($P<0.01$), and the heart rate increased 5±5% in the Adβ-gal animals (P=NS).

Physiologic Responses to Ventricular Rate Control

At the end of the experiment, Adβ-gal animals had extreme lethargy, obvious dyspnea when walking from the cage to the ECG/feeding area, increased sleep, loss of playful behaviors, and minimal appetites. At the time of sacrifice, these animals had negligible body fat, extensive ascites, and pericardial and pleural effusions.

Both wtGi and cGi groups had continued evidence of heart failure immediately after gene transfer, but the symptoms improved during the 18 days after gene transfer. The cGi group had no symptoms at the end of the study; they had normal activity and appetite. At the time of sacrifice, there was negligible extravascular fluid present. Of the animals in the wtGi group, 3 had mild heart failure symptoms with lethargy and mild dyspnea with exertion but a resumption of normal sleep and play patterns and no suppression of appetite. The other 2 animals had no detectable symptoms, but all wtGi animals had evident ascites and effusions at sacrifice.

Echocardiographic parameters were correlated with the clinical observations in the post–gene transfer period (the Table). β-Gal animals had increasing chamber sizes and worsening left ventricular function. The wtGi animals had modest improvements in left ventricular function and stabilization of ventricular chamber size but continued increases in left atrial size. The cGi group had near-normalization of left ventricular function, stabilization of left atrial and right ventricular chamber size, and a reduction in left ventricular size.

At the time of sacrifice, the hearts were examined for signs of intracardiac thrombus. One animal in the Adβ-gal group had a well-developed thrombus in the left atrium. No other animals in any group had intracardiac thrombus.

Histologic Findings After Gene Transfer

All hearts were subjected to histologic analysis with hematoxylin and eosin, Masson's trichrome, and TUNEL staining. The extent of cellular disorganization was more evident in ventricular tissue of control animals than in cGi animals, but signs of cellular hypertrophy and interstitial inflammation were similar for all groups. Left ventricular fibrosis was more extensive in β-gal and wtGi pigs than in cGi pigs (Figure 4). No significant differences were found between left ventricular samples of control and wtGi pigs, and no significant differences were noted for right ventricular samples. Despite the modest reduction in atrial size on the echocardiograms, no changes in any atrial histologic parameters were noted between groups.

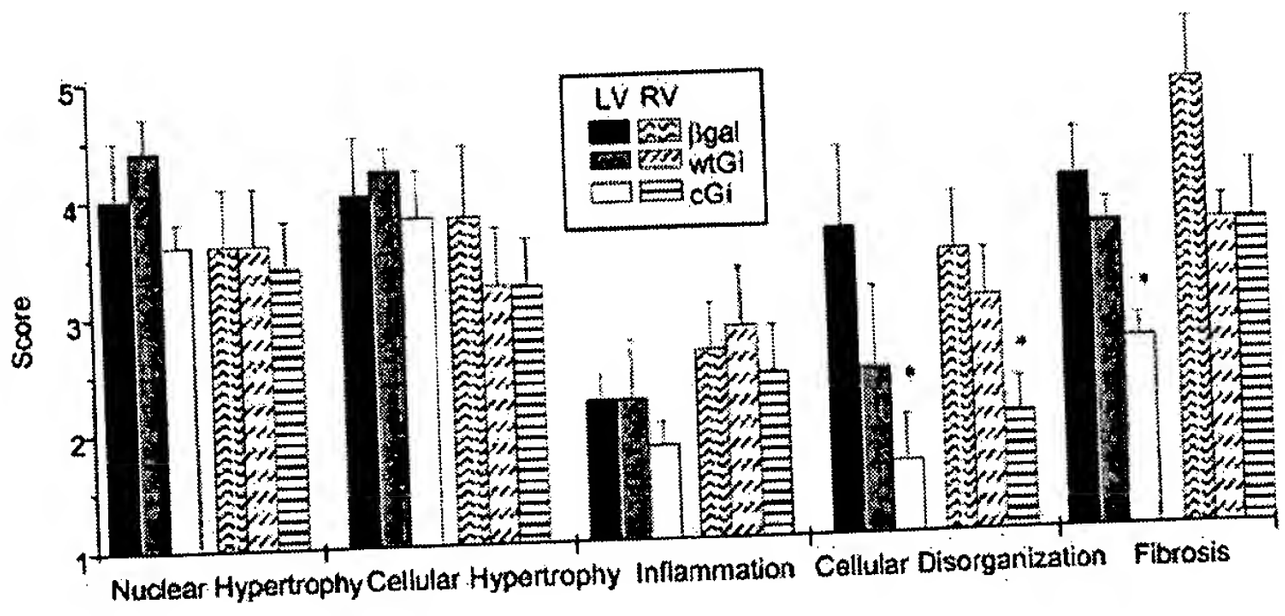

Figure 4. Results from hematoxylin and eosin and Masson's trichrome staining of cardiac samples. See text for details of scoring system. *$P<0.05$. LV and RV indicate left and right ventricle, respectively. All other abbreviations are as defined in text.

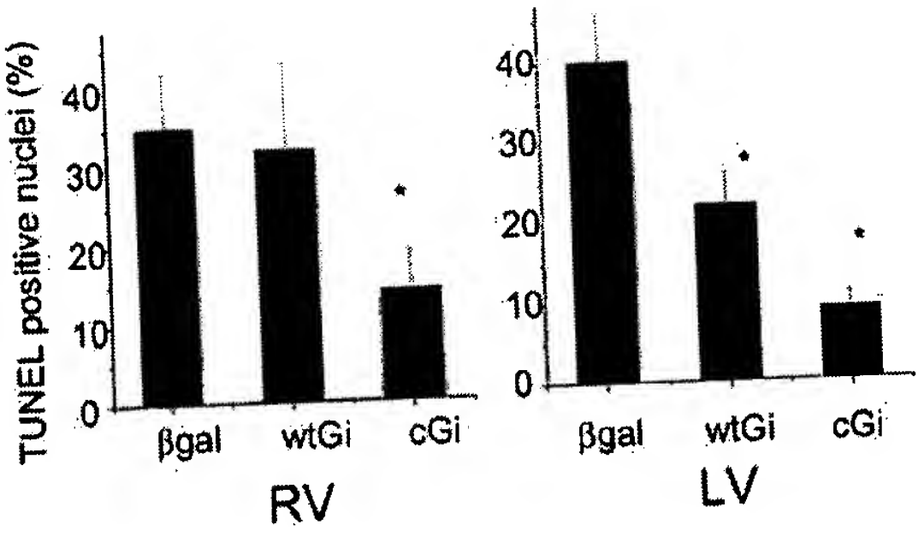

Figure 5. Results from TUNEL staining of cardiac samples. Animals with heart rate control after cGi overexpression had significantly lower numbers of TUNEL-positive nuclei compared with wtGi- or β-gal–expressing animals. *$P<0.01$. LV and RV indicate left and right ventricle, respectively. All other abbreviations are as defined in text.

Apoptotic activity was evaluated by TUNEL staining on left and right ventricular samples from all animals (Figure 5). TUNEL results in the left ventricle corroborated the echocardiography findings. cGi had the fewest TUNEL-positive nuclei, wtGi had an intermediate number, and β-gal had the most TUNEL-positive nuclei. In the right ventricle, cGi animals again had the fewest TUNEL-positive nuclei, but there were no differences between wtGi and β-gal groups. In all samples, the number of TUNEL-positive nuclei was elevated compared with controls, wherein TUNEL positivity was rare.

Discussion

Development of gene therapy as a viable treatment for cardiac arrhythmias has occurred at a disappointing rate. To date, there are only 3 in vivo examples of the concept: our original report of AV nodal gene transfer in sedated pigs with acute AF and 2 illustrations of genetic pacemaker activity.[7,16,17] In the current report, we continue the process of developing gene therapy as a potential option for cardiac arrhythmias. In a stepwise fashion, we first showed rate control in an acute model of AF,[7] and now we demonstrate rate control in a physiologically relevant model of persistent AF and severe chronic heart failure.

Relevance of the Model

Our original report of AV nodal gene therapy for ventricular rate control gave proof to the concept that gene therapy can treat common cardiac arrhythmias. A problem with that report was the somewhat contrived nature of the model. The pigs were anesthetized and instrumented at the time of heart rate measurement. The AF was created by short-term burst pacing and was self-limited in duration. The current report strives to test the hypothesis in a more clinically relevant model.

In patients, AF typically appears secondary to some other form of heart disease (eg, coronary artery disease, hypertension, valvular disease, or left ventricular dysfunction). Similar to the human condition, our porcine model had AF in the setting of cardiac dilation, dysfunction, and severe heart failure symptoms. Also as in many severe heart failure patients,[18] conventional drug therapy did not adequately control the ventricular response rate in our pigs. Another similarity is the tachycardia-induced left ventricular dysfunction, which occasionally plays a role in the human situation.[18] Major differences between the porcine model and the human condition are the extraordinarily fast ventricular rate and rapid development of cardiomyopathy in the porcine model, although it could be considered that the drug-refractory fast rate and severe left ventricular dysfunction make this an appropriately rigorous model for testing this new therapy.

Physiologically Relevant Rate Control With cGi Gene Transfer

cGi gene transfer to the AV node resulted in a 15% to 25% reduction in heart rate, resulting in reversal of the clinical symptoms, a moderate recovery of cardiac function, and the beginnings of cardiac structural remodeling. Both ejection fraction and left ventricular end-diastolic diameter improved significantly, whereas left atrial and right ventricular diameters stabilized. Most histologic parameters lagged behind the functional measurements, although the decrease in TUNEL-positive cells in the cGi group suggests that structural remodeling might have continued beyond the limited follow-up of this report. The exact mechanism for reversal of the tachycardiomyopathy is unclear and probably complex. In our original report, gene transfer was largely confined to the AV node, but there was a small area of gene transfer in the basal ventricular septum.[7] We cannot definitively exclude the possibility that gene transfer to this small region affected global ventricular function, but the temporal association between gene transfer, heart rate control, and functional improvement and the global nature of this improvement supports the idea that rate control is the primary factor in the reversal of tachycardiomyopathy. As such, our observed improvement in left ventricular function documents the physiologic relevance of the achieved level of ventricular rate control.

Combination of Heart Failure and Arousal Overcome the wtGi Effect

The response of wtGi in the persistent AF model was disappointing, given our previous report. The response does shed some insight into the interactions between physiologic status of the animal and transgene effects. During sedation, wtGi overexpression in the chronic AF/chronic heart failure model reduced heart rate to a level similar to that seen in the adrenergically stimulated animals of the acute AF study.[7] This result is consistent with the hyperadrenergic state previously documented in the AF/severe chronic heart failure model,[8] which also potentially explains the inefficacy of conventional AV node–blocking drugs in our model. When the chronic AF/chronic heart failure animals were awake, heart rate control was completely lost. This behavior is reminiscent of the effect seen with digoxin in humans, in whom rate control is adequate at rest but quickly lost during activity or adrenergic stimulation.[19] The interaction between adrenergic stimulation and wtGi overexpression suggests that the protein integrates normally into the G-protein receptor–coupled system. Obviously, further study is needed to inves-

tigate the mechanism on a molecular and cellular level, but the response to wtGi gene transfer provides encouragement that the gene transfer effect is caused by normal processing and placement of the transgene protein.

Conclusions

The current study continues the development of gene therapy as a potential option for this common arrhythmia. Our model is comparable to the clinical scenario of severe left ventricular dysfunction, persistent AF with a rapid ventricular response, and both inefficacy and intolerance of rate-controlling medications. Currently, treatment of such human patients requires ablation of the AV node and permanent dependence on a pacemaker. In our model of awake, fully functional animals, gene therapy–induced rate control was sufficient to reverse the tachycardia-induced cardiomyopathy. Further study with long-term expression vectors (eg, adeno-associated virus[20] or helper-dependent adenovirus[21]) and cardiac-specific promoters is needed, but this report advances the concept of using gene therapy as a future treatment option for common cardiac arrhythmias.

Acknowledgments

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Disclosure

Dr Donahue has ties to Excigen, Inc, a gene therapy company. None of the authors have any relationships to disclose. The observations made and measurements reported in the article were performed by Dr Bauer, who has no relationship to Excigen or any other gene therapy company. The statistical analyses were performed by Dr Nasir, who has no relationships to disclose.

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